



# An innovative water-assisted UV-C disinfection system to improve the safety of strawberries frozen under cryogenic conditions

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## ABSTRACT

Strawberries inoculated with *Salmonella enterica*, *Listeria monocytogenes* ( $10^8$  CFU/mL, 50  $\mu$ L) and murine norovirus (MNV-1;  $10^6$  TCID<sub>50</sub>/mL, 50  $\mu$ L), were washed for 2 min in a water-assisted UV-C light tank (WUVC) combined or not with 40 mg/L of peracetic acid (WUVC+PA), and 200 mg/L of free chlorine solution (NaClO) with the UV-C lamps switched off. Moreover, a 'conventional' dry UV-C treatment (DUVC) was also tested. After 2-min exposure, washing sanitization with chemical agents gave the highest reduction for both bacteria (ca.  $\geq 3.3$  log CFU/g) and MNV-1 ( $\geq 1.8$  log TCID<sub>50</sub>/mL). DUVC treatment proved to be the least effective technology ( $\leq 0.6$  log CFU/g for bacteria and 1.5 log TCID<sub>50</sub>/mL for MNV-1). Regarding wash water, no presence of *L. monocytogenes* and *S. enterica* were reported with WUVC+PA and NaClO sanitization. After disinfection, samples were frozen at  $-70 \pm 2$  °C in a cryogenic freezing cabinet with liquid nitrogen (N<sub>2</sub>). For both pathogens, frozen storage after washing substantially enhanced their inactivation in the first 3 days (1.1–4.9 log UFC/g) compared to the reductions obtained the following sampling points (0.0–0.8 log UFC/g). After 90 days, *L. monocytogenes* and *S. enterica* were not detected on the samples treated with water-assisted methodologies (WUVC, WUVC+PA and NaClO treatments), whilst MNV-1 was little affected. Further studies are needed to improve norovirus inactivation on frozen strawberries.

**Industrial relevance:** The present work provides relevant information to the frozen food industry regarding a suitable decontamination alternative to chlorine sanitation. Low-dose immersion-assisted UV-C allows inactivation and inhibition of pathogenic microbiota while generates non-toxic byproducts and allows reusing the process water, contributing to the so-called "smart green growth" attended to provide a more innovative and sustainable future for the food industry.

## 1. Introduction

The consumption of fruits and vegetables has increased worldwide, driven mainly by the changes in the life habits of people due to a growing concern for maintaining a more balanced diet (FAO, 2021; Fruitlogistica, 2020). For that reason, the consumption interests of European consumers have increased the demand of fruits such as strawberries, focused in the increasingly aware of their health benefits, rich in antioxidants and other biochemical compounds, which have been correlated to a reduced risk of heart problems and cancer disease (Battino et al., 2017; Giampieri et al., 2015; Wang, 2014). However, their elevated water content (ca. 90%) and high level of respiration make them vulnerable to microbial growth, mainly spoilage moulds, which results in a short shelf-life (1–2 days at room temperature) (Samadi,

Ghasemnezhad, & Imani, 2017; Tournas, Heeres, & Burgess, 2006; Wright & Kader, 1997). Therefore, strawberries are widely used in the food industry as an ingredient in other food products or principally as a frozen fruit in many regions of Europe, preventing its highly deterioration by microbial infection, with the availability to consume this product year-round (Haffner, 2002). Indeed, total production of frozen berries has been gradually increasing since 2015, with Europe being the largest market for frozen strawberries in the world, probably due to the popularity of healthy breakfast option (such as smoothies) (Dira, 2016). In 2017, it has been reported that the European consumers purchased around 1.2 tons of strawberries per year, being Spain in the 2nd place of the top-5 producers of frozen strawberries in Europe (CBI, Ministry of Foreign Affairs, 2019).

Even though frozen strawberries are very attractive for the

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consumers (Janowicz, Lenart, & Idzikowska, 2007), they have been linked to human norovirus and hepatitis A virus (HAV) foodborne disease outbreaks around the world in recent years (Baert et al., 2011; Bernard et al., 2014; Hjertqvist et al., 2006; Le Guyader et al., 2004; Maunula et al., 2009; Sarvikivi et al., 2012; Severi et al., 2015). In 2012, frozen strawberries were implicated in large-scale outbreaks of human norovirus and HAV. Approximately 11,000 people in Germany were affected by human norovirus gastroenteritis originated by frozen strawberries imported from China (Mäde, Trübner, Neubert, Höhne, & Johne, 2013) while HAV in frozen mixed berries (including strawberries) from various countries (Canada, Bulgaria, Serbia and Poland) was linked to an increase in cases in Northern Italy (Rizzo, Alfonsi, Bruni, Busani, & Ciccaglione, 2013). Moreover, at least 22 notifications related to the presence of viruses on berries have been reported since 2018 in the RASFF portal (RASFF, 2020). Last mentioned bibliography and recent publications clearly show that these viruses can survive and remain infectious after freezing conditions, remaining viable for periods exceeding the shelf-life of products (Bozkurt, Phan-Thien, Van Ogtrop, Bell, & Mcconchie, 2020; Butot, Putallaz, & Sanchez, 2008; Tavoschi et al., 2015). Survival of *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* were also plausible on strawberries during refrigeration, and frozen storage for at least one month (Flessa, Lusk, & Harris, 2005; Han, Linton, & Nelson, 2004; Knudsen, Yamamoto, & Harris, 2001; Yu, Newman, Archbold, & Hamilton-Kemp, 2001). For this reason, even though low temperatures minimize the respiratory rate in fruits, and the growth of pathogenic bacteria microorganisms, it is not considered as an effective mitigation strategy for enteric viruses on berries (Butot et al., 2018). This problematic has led to scientists and industries to find problem-solving approaches, mainly focused on introducing disinfection and sanitizing methods and preservation procedures in fruit produce workflow that sufficiently reduce levels of potential microbial contaminants, previous to frozen storage (Leistner, 2000). Indeed, some berries (strawberries and blueberries) picked for frozen processing are usually hulled in the field, transported to the processing facility, and washed with sodium hypochlorite (NaClO) solutions (50 to 200 ppm) to remove debris (e.g., twigs and rocks) with subsequent frozen storage, improving the advantages of freezing over refrigeration in terms of food safety and the extension of the fruit shelf-life (Bridges, Tadepalli, Anderson, Zhang, & Wu, 2019).

However, due to concerns regarding consumer and environmental safety of chlorinated washes, alternative methodologies have become research focuses (Collazo et al., 2018; Goodburn & Wallace, 2013). Other authors have studied the combination of alternative disinfectants (chloride dioxide, lactic acid and ozone) with freezing for the reduction of *Salmonella*, *L. monocytogenes* and *E. coli* on blueberries (Bridges et al., 2019; Tadepalli, Bridges, Driver, & Wu, 2018). Previously, our research group has studied the efficacy of a water-assisted short-wave ultraviolet (WUVC) technology, alone or combined with peracetic acid (PA), for the reduction of natural microbiota, *L. monocytogenes* and *S. enterica* in fresh strawberries (Nicolau-Lapeña et al., 2020; Ortiz-Solà, Abadías, Colás-Medà, Anguera, & Viñas, 2021; Ortiz-Solà et al., 2020), obtaining promising results and maintaining the fruit quality. To our knowledge, this combination technology has not been previously tested against enteric viruses on frozen strawberry produce.

Since the available information of enteric virus and foodborne bacteria survival on frozen produce, and the efficacy of the implementation of a washing step of current commercial processes for their removal or inactivation are still lacking, the aims of the present study were (i) to evaluate the efficacy of the combination of WUVC and PA coupled with an additional freezing step using a cryogenic cabinet freezer operated with nitrogen (N<sub>2</sub>), and (ii) determine the survival of *Salmonella enterica*, *Listeria monocytogenes* and murine norovirus (MNV-1), a human norovirus surrogate, on strawberries throughout one-year shelf-life at frozen storage (−25 °C).

## 2. Materials and methods

### 2.1. Fruit

Fresh strawberries (*Fragaria × ananassa*) were obtained from local providers in Lleida (Catalonia, Spain). Fruits were kept in trays overnight in air in the refrigerator (ERC-65, Infrico, Córdoba, Spain) at 4 ± 1 °C until use. Before the experiment, the peduncle of the fruit was carefully removed by hand. Only intact, healthy and same-sized (approximately 25 g) strawberries were selected.

### 2.2. Microbial strains, culture conditions and fruit inoculation

In the present study, a cocktail of five *Salmonella enterica* subsp. *enterica* strains: Agona (ATCC BAA-707; American Type Culture Collection, Manassas, USA), Michigan (ATCC BAA-709), Montevideo (ATCC BAA-710), Gaminara (ATCC BAA-711) and Enteritidis (CECT-4300; *Colección Española de Cultivos Tipo*, Burjassot, Spain), and five *Listeria monocytogenes* strains: serovar 1a (CECT-4031), serovar 3a (CECT-933); serovar 4d (CECT-940), serovar 4b (CECT-4032) and serovar 1/2a, which was previously isolated in our laboratory from a fresh-cut lettuce sample (Abadías, Usall, Anguera, Solsona, & Viñas, 2008), were used. *S. enterica* strains were grown individually in tryptone soy broth (TSB, Biokar Diagnostics, France) medium for 20–24 h at 37 ± 1 °C. *L. monocytogenes* strains were grown individually in TSB supplemented with 6 g/L of yeast extract (tryptone yeast extract soy broth, TSBYE) for 20–24 h at 37 ± 1 °C. Bacterial cells were harvested by centrifugation at 9800 ×g, 10 min at 10 °C. Identical content of the five *S. enterica* and *L. monocytogenes* were mixed to obtain a single suspension of *S. enterica* five-strain cocktail and *L. monocytogenes* five-strain cocktail.

The day before the experiment, fresh strawberries were inoculated with a suspension containing 10<sup>8</sup> CFU/mL of *L. monocytogenes* or *S. enterica* inoculum by pipetting 50 µL in small droplets on the fruit surface. Once dried (1–2 h at room temperature), strawberries were stored at 4 ± 1 °C overnight. Inoculum concentration was confirmed by plating appropriate dilutions onto XLD (Xylose-Lysine-Desoxycholate Agar, Biokar Diagnostics) for *Salmonella*, and onto PALCAM agar (PALCAM Agar Base with selective supplement, Biokar Diagnostics) for *L. monocytogenes*. The plates were incubated at 37 ± 1 °C for ca. 24 h for *Salmonella* and ca. 48 h for *L. monocytogenes*.

For the virus assay, murine norovirus (MNV-1), a human norovirus surrogate, was assessed. MNV-1 stocks were propagated at murine macrophage cell line RAW 264.7 (kindly provided by Prof. H. W. Virgin (Washington University School of Medicine, US)). Briefly, semi-purified MNV-1 virus was harvested 2 days after infection by three freeze-thaw cycles of infected cells followed by centrifugation at 660 ×g for 30 min to remove cell debris. RAW 264.7 cells were cultured in DMEM supplemented as described in Ortiz-Solà et al. (2020) and maintained at 37 ± 1 °C in a 5% CO<sub>2</sub> humidified incubator (NU-4950, NuAire, USA) in T175 flasks (Nunc, Thermo Fisher, USA) with 85% of relative humidity (RH).

Infectious viruses were enumerated by determining the 50% tissue culture infectious dose (TCID<sub>50</sub>) with eight wells per dilution and 20 µL of inoculum per well using the Spearman-Kärber method (Pintó, Diez, & Bosch, 1994). Stocks of MNV-1 (1 mL) were frozen until use (−80 °C). Frozen stocks were thawed and diluted one logarithmic unit (2.8 × 10<sup>7</sup> tissue culture infective dose TCID<sub>50</sub>/mL) with phosphate-buffered saline (PBS; ThermoFisher, US). Inoculation was done also as described above; fruits were allowed to dry and used the same day of inoculation.

Prior to the experiments, the initial concentration of *S. enterica*, *L. monocytogenes* and MNV-1 in the suspensions was checked as explained below.

### 2.3. Strawberry disinfection

#### 2.3.1. UV-C water-assisted tank equipment

All washing treatments were conducted in the UV-C water-assisted (WUVC) laboratory scale equipment LAB-UV-C-Gama (UV-Consulting Peschl, Castellón, Spain) equipped with 4 UV-C lamps (17.2 W), an aeration and recirculation system already described in Ortiz-Solà et al. (2020). Before and after each disinfection treatment, the temperature of water was measured using an infrared thermometer DualTemp Pro (Labprocess distribuciones, Barcelona, Spain) and the irradiance was measured through an orifice located in the lid of the equipment using a UV-sensor EasyH1 (Peschl Ultraviolet, Mainz, Germany).

#### 2.3.2. Sanitizing treatments

For each sanitization treatment, 20 strawberries (25-g each, approximately) were used. Samples were washed for 2 min using the WUVC equipment in combination with 40-mg/L (ppm) peracetic acid (PA) (WUVC+PA), since previous work reported that this combination significantly reduced pathogenic microorganisms on strawberries (Nicola-Lapeña et al., 2020). Moreover, WUVC alone and 200 ppm of sodium hypochlorite (NaClO) (pH 6.5) were also added as control treatments in the same equipment commented above. For WUVC and WUVC+PA treatments, lamps were preheated during 10 min until an irradiance of  $10.5 \pm 0.5 \text{ W/m}^2$  was achieved. The tank was filled with 12 L of cold tap water ( $8 \pm 2 \text{ }^\circ\text{C}$ ) with or without PA and recirculation and aeration system were switched on. Treatment time was set up for 2 min, corresponding to an irradiation dose of  $1.3 \text{ kJ/m}^2$ . For NaClO treatment, the process was set up at the same conditions except that the lamps were switched off. After NaClO treatment strawberries were rinsed in tap cold water for 2 min to eliminate any residual. The free chlorine concentration was checked with an ion specific meter Hanna Instruments HI 95734-11 (Rhode Island, US) and PA concentration was determined by iodometric titration with potassium permanganate and sodium hydroxide (NaOH) 2 M (Panreac AppliChem, Barcelona, Spain). Furthermore, pH and ORP (Oxidation Reduction Potential) values were measured using pH meter (Crison GLP-22, Barcelona, Spain). After the washing treatments, strawberries were let at room temperature to drain the excess of water (1–2 h at  $22 \text{ }^\circ\text{C}$ ).

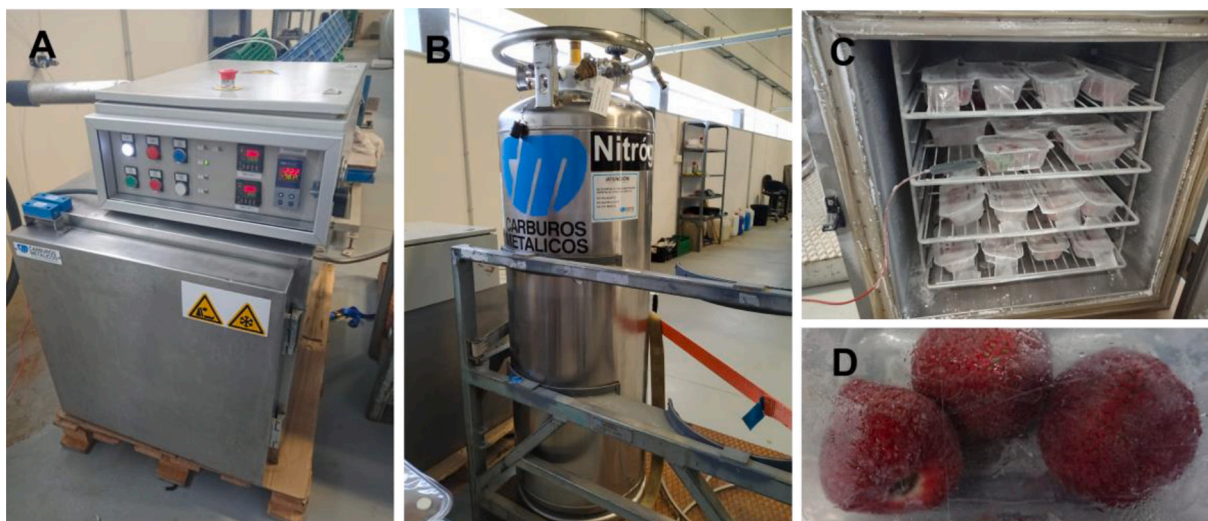
Moreover, one-sided dry UV-C (DUV) treatment without water immersion during 2 min was also tested ( $1.3 \text{ kJ/m}^2$ ) in an UV-C light cabinet, in order to compare it with the novel water-assisted UV-C technology. A batch of inoculated strawberries was left untreated (control, CK) for the comparison throughout the experimental time.

### 2.4. Strawberry cryogenic freezing process and storage conditions

Once dried, three strawberries per treatment ( $n = 3$ ) were packaged under polypropylene (PP) trays (375 mL) sealed with tray-lidding film by a self-sealing lab scale equipment (AK-RAMON TS-150, Barcelona, Spain). Packaged samples were frozen at  $-70 \pm 2 \text{ }^\circ\text{C}$  in a cryogenic freezing cabinet which operates by injecting liquid Nitrogen ( $\text{N}_2$ ) inside (Carburos Metálicos-Air Products Group Batch Freezer CM-85/1090, Carburos Metálicos SA, Barcelona, Spain, Fig. 1). With this equipment, freezing of fruits is done in three phases: (i) cooling from the initial fruit temperature to freezing point with freezing temperature (FT) (without crystal ice formation); (ii) super-cooling with a transition phase time with a remaining constant temperature (this is the phase in which most of the ice forms); (iii) super-chilling when the temperature falls for the mixture of water and ice, cooling down to a final temperature of approximately  $-20 \text{ }^\circ\text{C}$  (Freezing time (Ftime)). This methodology allows a smaller crystal formation, so cell integrity is maintained and water is better retained after thawing, offering potential benefits for the preservation of fresh foods (Comandini et al., 2013; Stonehouse & Evans, 2015; Sun, Pan, Pu, Zhu, & Han, 2019). In the present study, all trays including three strawberries per tray were first pre-cooled to approximately  $0\text{--}5 \text{ }^\circ\text{C}$  in the cryochamber commented above. Subsequently, strawberries were gradually cooled down to approximately  $-1.5 \text{ }^\circ\text{C}$  by the injection of liquid  $\text{N}_2$  (phase 2). During this cooling process, strawberries took the supercooling state. Finally, strawberries were frozen with a temperature of approximately  $-70 \pm 2 \text{ }^\circ\text{C}$ , to resolve the super-cooled state and freeze strawberries completely ( $-20 \text{ }^\circ\text{C}$ ). Throughout the whole freezing process, the internal temperature of the fruit was recorded by placing the probe of the data logger JUMO TDA-300/TDA-3000 (Berlin, Germany) in the interior of a single fruit. After freezing, packaged samples were stored at  $-25.0 \pm 0.5 \text{ }^\circ\text{C}$  for 12 months.

### 2.5. Microbiological analysis

Populations of *S. enterica*, *L. monocytogenes* and the infectivity of MNV-1 were evaluated before and after sanitation process and after 3, 90, 180 and 360 days of storage at  $-25 \text{ }^\circ\text{C}$ . Before and after each treatment, three strawberries were randomly taken and weighed. During storage, the day before each sampling time, three strawberries from the same tray ( $n = 3$ ) were taken. Each strawberry was placed in an individual sterile filter bag (80 mL BagPage®, Interscience BagSystem, Saint Nom, France) and samples were thawed at  $4 \text{ }^\circ\text{C}$  overnight. Fresh strawberries or defrost strawberries and exudates were diluted with



**Fig. 1.** (A) Cryogenic freezing cabinet (Carburos Metálicos – Air Products Group model Batch freezer CM-85/1090) which is cooled by injecting liquid Nitrogen ( $\text{N}_2$ ) from Carburos Metálicos (B). (C and D) Frozen strawberry samples after freezing process.



buffered peptone water 1:4 (w:v) and mashed in a paddle blender (MiniMix, Interscience, France) for 2 min at 9 strokes/s. Aliquots of the mixture were serially diluted in saline peptone (SP, plated (0.1 or 1.0 mL for fresh and frozen strawberries, respectively) in duplicate onto XLD for *S. enterica* and PALCAM agar for *L. monocytogenes* determination. Plates were incubated at  $37 \pm 1$  °C for 24 h and 48 h, respectively. Results were calculated as colony forming units per g (CFU/g) and expressed as log CFU/g. Frozen strawberries were left to thaw in the refrigerator (10 °C) overnight in sterile bags in order to maintain the exudates. Strawberries and their exudates were analyzed as previously described. For the samples analyzed after the decontamination and freezing steps, detection limit was 1.30 log CFU/g whilst that of samples during storage (90, 180 and 360 days) was 0.70 log CFU/g (5 CFU/g). Logarithmic reductions of the pathogens due to the washing treatments were calculated by the following equation (Eq. (1)):

$$\text{Log reductions (Log CFU/g)} = \text{Log } (N_0) - \text{Log } (N_t) \quad (1)$$

where  $N_0$  is the mean of the initial population, and  $N_t$  is the population at each sampling time (CFU/g).

Population of bacteria were also determined in wash water after the sanitation treatments. Duplicate 100 µL samples were plated in XLD and PALCAM as indicated above. In parallel, duplicate 1-mL samples were added to 9-ml Dey-Engley tubes. Results were expressed as log CFU/mL. When counts were below the limit of detection (50 CFU/mL), and presence was confirmed by Dey-Engley colour variation followed by streaking onto XLD or PALCAM, an arbitrary value of ½ limit of detection (25 CFU/mL) was attributed.

For MNV-1 determination, extraction of the virus from the treated samples ( $n = 3$ ) was performed as previously described (Ortiz-Solà et al., 2020). Briefly, confluent RAW 264.7 cells with supplemented DMEM 10% were transferred to 96-well microtiter plates (ThermoFisher, USA). Micro-plates were stored at  $37 \pm 1$  °C in a 5% CO<sub>2</sub> and 85% of humidity relative (HR) conditions during  $24 \pm 2$  h. Afterwards, DMEM 10% was removed and 20 µL of the tenfold dilutions with PBS of each extracted sample were inoculated into 8 wells of a 96-well microtiter plates of confluent RAW 264.7. Plates were incubated at same conditions commented above. After 1 h incubation, 150 µL/well of DMEM supplemented with 2% FBS were added and incubated for 2–3 days at 5% CO<sub>2</sub> and 85% of HR. Then, RAW 264.7 monolayers with cytotoxicity effects were observed by visual examination using the optical inverse microscope. MNV-1 positive sample was diluted one log in PBS (2 M NaNO<sub>3</sub>, 1% beef extract, and 0.1% Triton X-100) and used as norovirus control. Negative controls were studied using PBS.

The MNV-1 infectivity of each treated strawberry was calculated by determining TCID<sub>50</sub> with 8-wells per dilution and 20 µL of inoculum/well. The number of wells with cytopathic effect were documented. The reduction of the infectivity was calculated as  $\log(N_0/N_t)$ , where  $N_t$  is the infectious virus titer after each treatment and  $N_0$  is the initial virus infect titer found in untreated strawberries (Falcó et al., 2018).

## 2.6. Statistical analysis

All data were analyzed for significant differences by applying analysis of variance test (ANOVA). The criterion for statistical significance was  $P < 0.05$  with the Tukey's Honest Significant Difference (HSD) test to evaluate the differences during storage conditions after the disinfection treatments. All statistical analyses were carried out using JMP PRO 14.0.1 (SAS Institute Inc., Cary, USA). MNV-1 experiment was repeated twice with 3 replications ( $n = 6$ ).

## 3. Results and discussion

### 3.1. Foodborne pathogenic bacteria inactivation on strawberries and wash water after disinfection treatments

Parameters of the water used to wash strawberries were analyzed during each sanitization treatment (Table 1). There were no differences between pH, ORP, peracetic acid (PA) and chlorine concentration after 2 min treatment (data not shown). Regarding the effect of the assayed technologies on microbial populations in the process water after washing for 2 min, foodborne bacterial pathogens were only found in the water-assisted UV-C (WUVC) treatment without PA, persisting in populations of  $<1.0$  log CFU/mL for both microorganisms. On the other hand, no presence of *L. monocytogenes* and *S. enterica* were reported in wash water after the WUVC combined with PA at 40 ppm (WUVC+PA), and after chlorine (NaClO) sanitization at 200 ppm. Undoubtedly, bacterial cells that are washed off from the fruit product are inactivated by the sanitizer present in the wash solution, thereby reducing the risks for cross contamination. The three-way action for disinfecting the produce by the synergistic effect of integrated strategies involving UV-C light, PA, and the simultaneous physical movement of the wash water could account for the higher efficacy against foodborne pathogens in washing water and fruit (as could be seen below). Compared to other wash water disinfectants, PA has less potential of producing degradation by-products, which are easily dissolved in water and non-toxic, thus making this sanitizer a good alternative to chlorine (Banach, Sampers, & Van Haute, van der Fels-Klerx, 2015).

On strawberries, initial counts of *S. enterica* and *L. monocytogenes* population were  $5.7 \pm 0.4$  and  $5.6 \pm 0.2$  log CFU/g, respectively (Fig. 2A and B). After 2-min exposure to disinfection treatments, NaClO sanitization and the combination of WUVC+PA gave the highest reduction of both bacteria (ca.  $\geq 3.3$  log CFU/g). On the other hand, the non-immersed dry UV-C (DUVC) technology ( $1.3$  kJ/m<sup>2</sup>) gave reductions about 0.4 and 0.6 log CFU/g for *S. enterica* and *L. monocytogenes*, respectively, being equivalent to the population obtained with the untreated samples (CK) ( $P > 0.05$ ). This lower inactivation observed with air-transmitted DUVC treatment were in concordance with previous investigation, reporting  $<1$  log reduction of *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* on fresh strawberries after 120 s exposure ( $13.3$  kJ/m<sup>2</sup>) of DUVC (Butot et al., 2018). On the other hand, bacterial population were reduced to 2.6 and 2.2 log for *L. monocytogenes* and *S. enterica* when strawberries were washed with WUVC alone, while the addition of 40 ppm of PA increased the reduction range to 1.0 and 2.0 log CFU/g, respectively. However, there were no significant differences between them ( $P > 0.05$ ). Therefore, results confirmed the effectiveness

**Table 1**

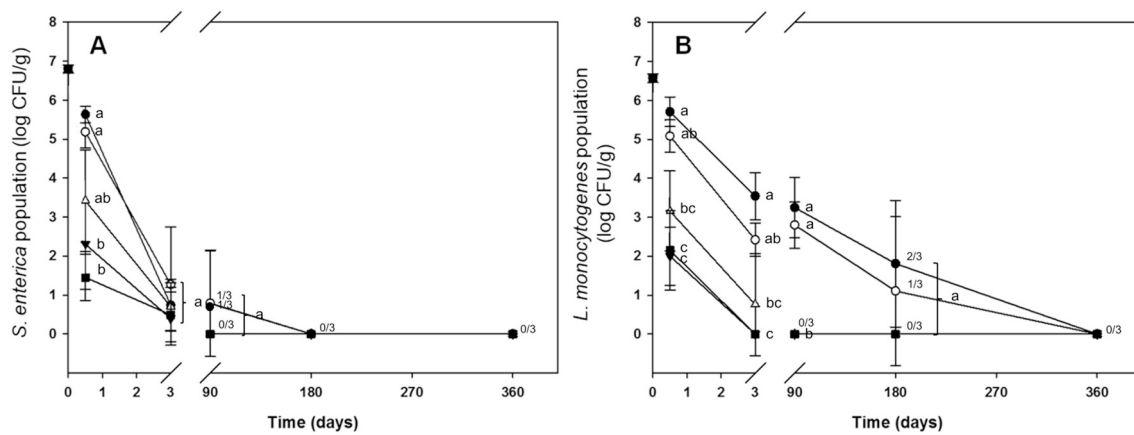
Water parameters: pH, ORP, concentration of sanitizer and *L. monocytogenes* and *S. enterica* population in washing water after 2 min exposure. Pathogenic bacteria population values are the mean of the 4 repetitions  $\pm$  standard deviation.

| Treatment <sup>1</sup> | [] of free chlorine or PA (mg/L) | pH                  | ORP (mV)                     | <i>L. monocytogenes</i>            | <i>S. enterica</i>                 |
|------------------------|----------------------------------|---------------------|------------------------------|------------------------------------|------------------------------------|
| WUV-C                  | $<0.01$                          | 7.7<br>$\pm$<br>0.6 | 201.5<br>$\pm$<br>$\pm 12.1$ | $0.80 \pm 0.59$ (3/4) <sup>2</sup> | $0.72 \pm 0.52$ (3/4) <sup>2</sup> |
| WUV-C + PA             | $40.7 \pm 2.7$                   | 5.3<br>$\pm$<br>0.2 | 465.0<br>$\pm$<br>$\pm 2.8$  | ND*                                | ND*                                |
| NaClO                  | $184.0 \pm 3.5$                  | 6.7<br>$\pm$<br>0.1 | 879.5<br>$\pm$<br>$\pm 2.1$  | ND*                                | ND*                                |

ND\*: no-detected in the wash water.

<sup>1</sup> WUV-C: water-assisted ultraviolet disinfection, WUV-C + PA: ultraviolet disinfection combined with peracetic acid, NaClO: hypochlorite solution.

<sup>2</sup> Number samples that were confirmed positive after enrichment in Dey-Engley/total samples analyzed



**Fig. 2.** Population (log CFU/g) of *Salmonella enterica* (A) and *Listeria monocytogenes* (B) as a function of the applied disinfection treatments and storage time ( $n = 3$ ). CK: control samples (without washing) (●), DUVC: conventional dry ultraviolet disinfection (○), NaClO: hypochlorite solution (200 ppm) (▼), WUV-C: water-assisted ultraviolet disinfection (△), WUV-C + PA: water-assisted ultraviolet disinfection combined with peracetic acid (40 ppm) (■). For each represented time, different letters indicate significant differences among disinfection treatments according to the HSD Tukey post-hoc test ( $p < 0.05$ ). Numbers in fraction represent the number of samples testing positive after enrichment out of the total analyzed samples (3).

of the different washing treatments, since the WUVC and the combination treatment (WUVC+PA) are comparable to disinfection with free chlorine ( $P > 0.05$ ), allowing the inhibition of the principal foodborne bacterial pathogens on fresh strawberries after the different decontamination methodologies tested during 2-min exposure (Nicolau-Lapeña et al., 2020; Ortiz-Solà et al., 2020).

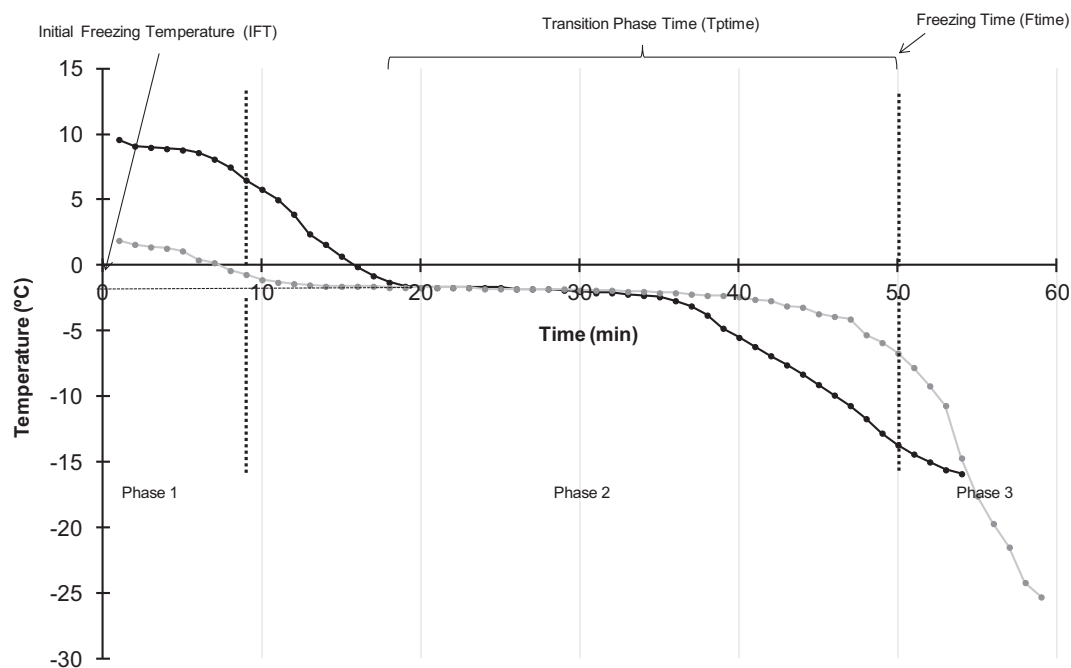
### 3.2. Foodborne pathogenic bacteria survival after cryogenic freezing process of strawberries during shelf-life

Freezing curves and phases of strawberry samples inoculated with *L. monocytogenes*, *S. enterica* and murine norovirus (MNV-1) frozen at  $-70\text{ }^{\circ}\text{C}$  in the cryogenic freezing cabinet are represented in Fig. 3. The process observed using cryogenic technology has been the typical freezing process used to freeze food at an extremely fast rate in the food

industry (Comandini et al., 2013).

Cryogenic freezing process, evaluated after 3 days of storage at  $-25\text{ }^{\circ}\text{C}$ , resulted in a sharp reduction of *S. enterica* (Fig. 2A) regardless of the disinfection treatment carried out before freezing, resulting in a final population of  $<1.3\text{ log CFU/g}$  in all treatments ( $P < 0.05$ ). Therefore, the inactivation achieved during the frozen storage was sometimes rather substantial compared to the inactivation caused by the disinfection treatments used. In fact, reductions  $>4\text{ log units}$  were attributed to the freezing process for *S. enterica* in the untreated samples. On the contrary, cryogenic freezing did not affect in such a way the viability of *L. monocytogenes* (Fig. 2B), with reductions of 2 log approximately for all treatments, only with remaining populations about  $<1.0\text{ log}$  on the surface of frozen strawberries treated with washing procedures.

For both pathogens, frozen storage at  $-25\text{ }^{\circ}\text{C}$  showed a slower but steady decline of bacterial counts during the following 90 days. This



**Fig. 3.** Freezing parameters and curves of strawberry samples inoculated with *Listeria monocytogenes* and *Salmonella enterica* (black) and murine norovirus (MNV-1) (grey) frozen at  $-70\text{ }^{\circ}\text{C}$  in a cryogenic freezing cabinet comprising liquid nitrogen ( $\text{N}_2$ ), from the brand Carbueros Metálicos – Air products Group model Batch freezer CM-85/1090.

survival behaviour of *L. monocytogenes* and *S. enterica* on frozen strawberries was similar to previous investigations, which reported that a freezing step had a greatest impact on *L. monocytogenes* and *E. coli* O157:H7 within the first 24 h of storage at  $-20\text{ }^{\circ}\text{C}$ , when populations in strawberry samples without added sucrose decreased by almost 1 log cycle. However, the short-term (30-day) survival of both pathogens was generally constant on frozen strawberries (Flessa et al., 2005; Knudsen et al., 2001). On the other hand, the tailing effect observed in the survival curve could be due to differences in resistance to acid/frozen storage among the strains included in the 5-strain cocktail (Huang, Ye, & Chen, 2013). It was previously demonstrated that antimicrobial washing (chorine, chlorine dioxide and lactic acid) combined with freezing step significantly reduced levels of *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. monocytogenes* in blueberries compared with what washing alone was capable of achieving in maximum log reduction. In this study, wash treatments alone resulted maximum log reductions from 0.4 to 2.0, while additional freezing step increased this to a range from 1.7 to 4.4 log (Tadepalli et al., 2018). Similarly, Bridges et al. (2019) evaluated the efficacy of different antibacterial washes coupled with frozen storage against foodborne pathogens on blueberries, which were treated with sodium hypochlorite (NaClO, 200 ppm), chlorine dioxide (15 ppm), ozone (3 and 5 ppm), or lactic acid (2%) for short exposure times (10 s, 1 min, or 3 min) with an additional freezing hurdle at  $-12\text{ }^{\circ}\text{C}$ . They found that wash treatments alone resulted in maximum log reductions from 1.0 to 2.8, while the additional freezing step increased this to a range from 3.7 to 6.6 after 1 week of storage. The greatest reduction of *L. monocytogenes* (6.6 log) and *Salmonella* Typhimurium (5.3 log) was observed after freezing combined with 3 min of exposure to 2% lactic acid or 200 ppm of NaClO, respectively (Bridges et al., 2019).

After 90 days at frozen storage, *L. monocytogenes* and *S. enterica* were not detected on the samples treated with water-assisted methodologies

(WUVC, WUVC+PA and NaClO treatments). However, the remaining population of *L. monocytogenes* was  $2.8 \pm 0.6$  and  $3.3 \pm 0.8$  log CFU/g when the samples were treated with non-immersed DUVC treatment and untreated strawberries, respectively. *S. enterica* was detectable by enrichment with 1 out of 3 samples being positive ( $<5$  CFU/g) after 90 days in the samples treated with DUVC. The results with bacteria on the surface of the fruit yielded further decreases in *S. enterica* populations on untreated samples (CK) throughout the experimental time, with reductions ca. 4-log compared to the initial level. Nonetheless, *L. monocytogenes* population was maintained on CK samples after 180 days ( $1.8 \pm 1.6$  log CFU/g). These results showed that *L. monocytogenes* could survive on the surface of the frozen fruit, although the populations declined markedly. Indeed, frozen foods do not support *L. monocytogenes* growth while kept at freezing temperatures, but can survive for extended periods on other food matrix (e.g., for 120 days at  $-18\text{ }^{\circ}\text{C}$ ) on whole and cut cucumbers (Bardsley et al., 2019) or at least 28 days at  $-20\text{ }^{\circ}\text{C}$  on whole and cut strawberries (Flessa et al., 2005) particularly in low acid food, including vegetables (Palumbo & Williams, 1991). Even if some cell injury may occur, the reduction of the pathogen during the frozen storage of low acid fruits and vegetables is very limited (ca. 1 log<sub>10</sub> in 100 days) (Pappelbaum et al., 2008).

After one-year of frozen storage, both pathogens were completely inactivated. Even starting for populations that are rather unrealistic, the use of WUVC and WUVC+PA before freezing decreased the population of *L. monocytogenes* below 100 CFU/g (that allowed by EU Regulation N° 2073/2005 and further amendments) after 3 days of storage.

### 3.3. Effect of the sanitizing treatments on the infectivity of MNV-1 on strawberries after sanitation procedures and cryogenic freezing step

The initial virus titer of artificially inoculated strawberries was  $3.4 \pm$

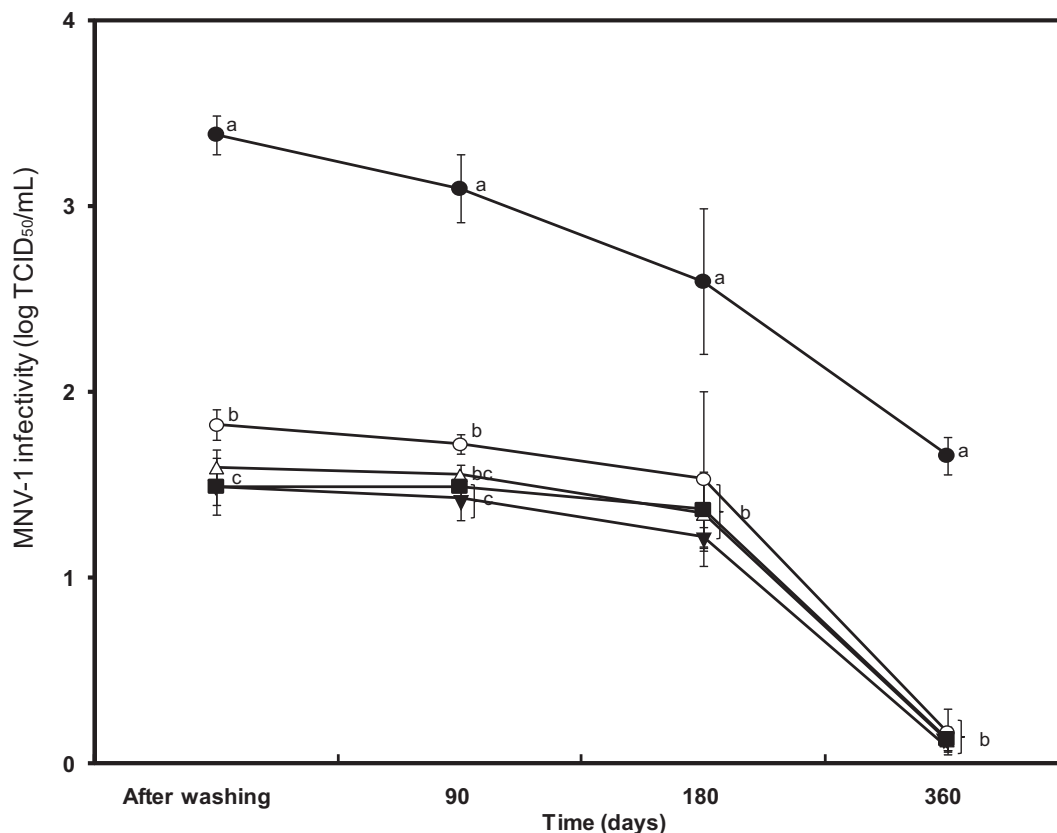


Fig. 4. Infectivity (log TCID<sub>50</sub>/mL) of the MNV-1 in relation to the applied disinfection treatments and different storage time ( $n = 6$ ). CK: control samples (without washing) (●), DUVC: conventional dry ultraviolet disinfection (○), NaClO: hypochlorite solution (200 ppm) (▼), WUVC: water-assisted ultraviolet disinfection (△), WUVC + PA: water-assisted ultraviolet disinfection combined with peracetic acid (40 ppm) (■).

0.1 log TCID<sub>50</sub>/mL. After 2-min of disinfection treatments, the infectivity of MNV-1 was significantly lower compared with that obtained with the untreated samples (CK) (Fig. 4). Likewise pathogenic bacteria, washing procedures are needed for the effective inactivation of MNV-1 infectivity, since DUVC treatment proved to be the lowest effective technology. Reductions obtained with the water-assisted sanitization treatments ranged between 1.8 and 1.9 log TCID<sub>50</sub>/mL, whereas those obtained with the DUVC methodology did not exceed 1.5 log TCID<sub>50</sub>/mL ( $P < 0.05$ ).

The infectivity of MNV-1 after cryogenic freezing process remained in frozen storage strawberries ( $-25^{\circ}\text{C}$ ) after 90 days, with no significant differences from the titer obtained after disinfection step (Data not shown). In fact, Butot et al. (2008) observed that HAV and rotavirus remained unaffected in unwashed fresh strawberries, raspberries, and blueberries, despite 90 days in frozen storage. Baert et al. (2008) reported that no reduction of MNV-1 on spinach and spring onions over 180 days of frozen storage. Unlike bacterial pathogens, maintaining the cold chain is not a mitigation strategy for viral pathogens on fresh produce, as persistence of enteric viruses is higher at low temperatures and inactivation rates generally increase with the increasing temperatures (Li, De Keuckelaere, & Uyttendaele, 2015). It is not surprising that MNV-1 used in the present study was little affected by freezing, as the results of our study corroborate data from documented outbreaks involving HAV and human norovirus and linked with frozen berries (Cotterelle et al., 2005; Falkenhorst et al., 2005; Hjertqvist et al., 2006; Hutin et al., 1999; Korsager, Hede, Boggild, Bottiger, & Molbak, 2005). Indeed, MNV-1 survived even the harsh conditions of cryogenic freezing with low pH of fruit. It has been demonstrated that MNV-1 had tolerance to a low pH (pH 2 for 1 h; Li et al., 2015; Verhaelen et al., 2013).

After 180 days, the infectivity of MNV-1 on untreated strawberries (CK) decreased, but remained statistically higher compared with the frozen strawberries treated with the studied technologies that were equally effective among them ( $P > 0.05$ ). Results obtained with norovirus, showed again that the disinfection step is needed, since infectivity of MNV-1 decreased after 360 days by  $>1.6$  log for both untreated and disinfected samples, with a remaining infectivity of ca. 1.5 and 0.1 log TCID<sub>50</sub>/mL, respectively. However, even low infectivity of MNV-1 was found on strawberries after 360 days of storage, some investigations suggest that only 18 to 1000 virus particles of human norovirus are necessary to cause the disease (Teunis et al., 2008).

The results of the present study reported that WUVC and WUVC+PA treatments were equally effective than 200 ppm of NaClO sanitization during experimental time ( $P > 0.05$ ). It is well known that biocides that have activity against both enveloped and non-enveloped viruses include chlorine releasing agents, peracids and ozone. Their effectiveness depends on the nature of the virus, the surface carrier, the presence of interfering substances such as organic soil or hard water salts, and contact time (Vasickova, Pavlik, Verani, & Carducci, 2010). On the other hand, the mechanism involved in antiviral activity of UV-C is probably the disruption of viral structure that ultimately degrades viral proteins and RNA (Woo et al., 2019). Previous studies reported that subsequent UV-C decontamination after other control strategies (e.g., chlorine or peracetic acid) resulting in synergistic benefits that could lead to increased UV-C induced viral genome damage (Rattanakul, Oguma, & Takizawa, 2015). The effectiveness of UV-C light-based technologies is limited due to the shadow effect. For this reason, the incorporation of water to the treatment enhanced the efficacy of this innovative technology. Turbidity of the wash water used could affect UV-C penetration (Abadias, Colás-Medà, Viñas, Bobo, & Aguiló-Aguayo, 2021) and to extend exposure times for better UV-C efficacy may not be realistic and should be further studied. Successful application of this combined technology relies on the light reaching all the virus particles directly and, if the viruses are present in cracks, crevices or openings in the surface of the food or surfaces, they could be inactivated with the chemical sanitizer present in the water.

#### 4. Conclusions

Results from this study indicated that when the antimicrobial disinfection is combined with freezing, injured bacterial cells that survived the washing step can be eliminated by exposure to this second hurdle over time. The subsequent use of these frozen strawberries in retail food operations or at home is particularly important when frozen berries are used in preparation of smoothies, milkshakes and other foods not intended to go through a thermal process. Moreover, we also showed that further research is warranted to address specific questions regarding the effectiveness of disinfection step coupled with frozen storage against MNV-1, since human norovirus is a highly stable virus that can survive from multiple days up to months on strawberry surfaces at freezing conditions, making essential the introduction of a washing step in strawberry processing. On the other hand, the amount of wastewater generated per mass unit of product depends on the disinfection technique employed, so UV-C irradiation being capable of disinfecting efficiently both the process water and the product, a higher ratio of recycling can be achieved, with a lower impact on the environment.

#### Declaration of Competing Interest

The authors declare no conflict of interests.

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